DIFFERENTIAL RECRUITMENT OF HOST PROTEINS TO THE *COXIELLA BURNETII* VACUOLE IN THE ABSENCE OF THE STEROL REDUCTASE CBU1206

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DEDICATION

“Life's most persistent and urgent question is: 'What are you doing for others?”

-Dr. Martin Luther King, Jr.

It is my deepest and sincere desire that this work may serve as a stepping stone for the scholarship of others and myself

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"The influence of a good teacher can never be erased"

I would like to acknowledge my mentor who embodied the word

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Rochelle Chashmi Ratnayake

DIFFERENTIAL RECRUITMENT OF HOST PROTEINS TO THE *COXIELLA BURNETII* VACUOLE IN THE ABSENCE OF THE STEROL REDUCTASE CBU1206

Q fever is a heavily underdiagnosed and underreported infection caused by the obligate intracellular pathogen *Coxiella burnetii.* Following entry into the host cell, *Coxiella* replicates in the acidic phagolysosome-like parasitophorous vacuole termed the *Coxiella* Containing Vacuole (CCV). The CCV is a large and highly fusogenic compartment that actively fuses with the host endocytic pathway during maturation of the phagolysosome. Evidence suggests that the development of the CCV is sensitive to increasing cholesterol levels and leads to CCV acidification and bacterial death. Therefore, we hypothesize that CCV cholesterol concentration is carefully modulated through the *Coxiella* encoded sterol reductases (CBU1206 and CBU1158). A *ΔCBU1206* mutant of *Coxiella* is hypersensitive to cholesterol and displays growth defects in intracellular replication and CCV development.

Following fusion with the host endocytic pathway, the *Coxiella* NMII Phase II (WT) CCVs readily acquire host proteins such as LAMP1, CD63, Rab7, ORP1L, RILP, and LC3. These heterotypic events with the host endosomal cascade are presumed to provide selected subsets of endocytosed cargo and membrane. Therefore, I investigated whether *ΔCBU1206* CCV heterotypic fusion events are defective due to altered lipid content on the CCV membrane.

I observed increased accumulation of sterols on the *ΔCBU1206* CCV membrane. Similar to WT, the mutant readily fuses host lysosomes and readily acquires the host glycoprotein LAMP1 but displays reduced localization of CD63 (LAMP3). Additionally, reduced localization of the late endosomal markers Rab7, ORP1L, and RILP was observed suggesting that late endosome fusion maybe defective in *ΔCBU1206*. Further, reduced localization of LC3 was also observed suggesting that the mutant may also be defective in fusing with autophagosomes. Finally, the mutant possesses a functional Type 4 Secretion System that secretes a moderate amount of effector proteins relative to WT. Considering the vast array of functions accomplished by the effectors secreted,

the moderate effector secretion by the mutant could influence the endocytic pathway fusion processes as well as CCV development. Collectively, this body of work suggests that the lack of sterol reductase CBU1206 in *Coxiella* results in defective heterotypic fusion events of the CCV membrane that could alter pathogenesis and CCV expansion.

Stacey Gilk, Ph.D., Chair

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LIST OF ABBREVIATIONS

ACCM 2	acidified citrate cysteine medium-2
AnkG	ankyrin repeat
ARD	ankyrin repeat domain
cAMP	cyclic AMP
CCV	*Coxiella* containing vacuole
CERT	CERamide Transfer
CR3	complement receptor-3
CyaA	Adenylate cyclase
DHCR24	24-Dehydrocholesterol Reductase
DotA	secreted component of T4SS
dpi	days post infection
EE	early endosome
EEA1	Early Endosome Antigen 1
ER	endoplasmic reticulum
GAPs	GTPase-activating proteins
GEFs	guanine nucleotide exchange factors
hoCT2	cation transporter 2
hpi	hours post infection
IgA1	immunoglobulin A1
LAMP1	Lysosome-associated membrane glycoprotein-1
LC3	Microtubule-associated protein 1A/1B-light chain 3
LCV	large-cell variant
MCS	Membrane Contact Sites
MNGC	multinucleated giant cell formation
ORD	OSBP-related domain
ORP1L	Oxysterol-binding protein-related protein 1
OSBP	Oxysterol-binding protein
Rab7	Ras-related protein Rab-7a

RILP	Rab-interacting lysosomal protein
SCV	small-cell variant
SNARE	Soluble NSF Attachment Protein Receptor
SLAPs	Spacious Listeria-containing Phagosomes
T4SS	Dot/Icm type IV secretion system
TGN	Trans Golgi Network
VAP	Vesicle Associated Protein

INTRODUCTION

History of Q Fever

In 1935, an outbreak of a mysterious undiagnosed febrile illness occurred among slaughterhouse workers in Brisbane, Australia. One year later, Herald Rea Cox and Gordon Davis of the Rocky Mountain Laboratory unknowingly isolated the same infectious agent from ticks near Nine Mile Creek in Missoula, Montana [1]. They described it as a "filter-passing" microorganism and called it *Rickettsia diaporica*.

At the time, the causative agent and the disease were not well characterized and was termed "Query" fever and is now commonly referred to as "Q fever". Continuing to investigate the mysterious illness, Edward Holbrook Derrick, the Director of the Laboratory of Microbiology and Pathology in the Queensland Health Department at Brisbane, attempted to isolate the agent of disease [2]. He induced the illness in guinea pigs but was unable to isolate the bacterium and suspected the culprit to be a virus. Although this initial attempt at isolating the pathogen was unsuccessful it was later reattempted by Frank Macfarlene Burnet and Mavis Freeman who were successful at isolating the bacterium from patients [3]. Their work on the spleen sections of infected mice used the Giemsa method to visualize miniature rods. Taking into account the small size of the rods and the vacuoles that were different from any known type of Rickettsia, the authors concluded that they were observing a new type of Rickettsia and named it *Rickettsia burnetii* [4]. In 1938, the first reported laboratory-acquired Q fever case occurred in Rolla Eugene Dyer, the Director of the National Institutes of Health [2]. Thus, the link between the Nine Mile agent from Montana and the Australian agent of Q fever was established when cross-immunity was demonstrated between the two. Once the causative agent was definitively established and the pathogen was discovered to be significantly different from other organisms in the Rickettsia genus; the pathogen was renamed *Coxiella burnetii* to acknowledge both research groups [5]

Coxiella Infection in Mammals

C. burnetii is commonly found in goat, sheep, and cattle reservoirs but has been confirmed in marine mammals, reptiles, and birds [6]. Most infections in animals are

asymptomatic and causes reproductive disorders ranging from abortion, endometritis, low birth weight, and infertility. The main route of *C. burnetii* transmission is by inhaling aerosols contaminated with the organisms and these aerosols are frequently shed from infected animals [7]. Shedding primarily occurs through birth products, milk, urine, and feces of infected animals; however, animals have seroconverted after exposure to *C. burnetii* without any active shedding of organisms. Humans tend to acquire the infection by inhaling contaminated aerosols and consumption of contaminated raw milk and dairy products. The pathogen preferentially infects cell types that rapidly internalize pathogens such as mononuclear phagocytes and macrophages [8]. Symptoms of *C. burnetii* infection include fever, fatigue, chills, and myalgia occur following an incubation period of 2 to 3 weeks [7] [6].

Approximately 60% of the infections are reported to be asymptomatic while the remaining patients acquire acute Q fever that manifests through an influenza-like illness, atypical pneumonia or hepatitis [9]. While only 1% to 5% of Q fever cases progress to chronic infection, these cases can result in fatal endocarditis that can occur months to years following acute infection. Chronic Q fever can also result in chronic fatigue syndrome characterized by myalgia, night sweats, arthralgia, night sweats, and changes in moods. In pregnant women Q fever has been shown to cause placentitis, abortion, low birth weight, premature birth, and consequently neonatal death [10]. Similar to reports of other mammals infected with *C. burnetii*, pregnant women with Q fever suffer from bacterial colonization that can occur in the uterus, placenta or mammary glands. Chronic complications such as these tend to occur if the woman is pregnant at the time of primary infection [9].

The largest *C. burnetii* outbreak to date occurred in the Netherlands where there were 4000 human cases between 2007 and 2009. There were a series of community outbreaks that occurred in the south-eastern provinces of the Netherlands and goats were identified as the primary source of the animal outbreaks. During the epidemic 28 dairy goat farms and 2 dairy sheep farms reported abortion percentages upwards of 60% [11].

The Bacterium

C. burnetii is a pleomorphic Gram-negative coccobacillus 0.2 to 0.4 µm and approximately 1 µm long but not stainable with the Gram technique [12]. The bacterium is known to double every 20 and 45 h inside cells where it forms a vacuole that progressively acquires phagolysosome-like characteristics. *C. burnetii* is the only bacterial pathogen that actively replicates inside a phagolysosome-like vacuole, which is known as the *Coxiella* Containing Vacuole (CCV). The vacuole is characterized by its acidic pH, cationic peptides and acidic hydrolases [13]. *Coxiella* is able to transition between two cell variants: the large-cell variant (LCV) and the small-cell variant (SCV). The LCV is metabolically active while the SCV is environmentally stable. SCVs are smaller rods approximately 0.5 µm in size and are characterized by a condensed cytoplasm and condensed chromatin. This environmentally stable cell variant form is resistant to sonication, heat shock, osmotic shock, oxidative stress, and pressure. This form is also known to express Hq1 and ScvA, basic peptides that are predicted to function in DNA binding during periods of extended dormancy [14, 15]. In contrast, LCVS are the replicative form that is typically larger than 0.5 µm and contain dispersed chromatin [12]. Translation machinery of the bacteria such as elongation factor Ts (EF-Ts) and elongation factor Tu (EF-Tu) were shown to be upregulated in LCVs and not in SCVs [16]. The transition of the bacteria from SCV to LCV is thought to occur approximately 8 hours post infection (hpi) but the CCV is predominantly LCVs later on in the infection around 2 to 6 days post infection [17]. These differences between the SCV and LCV forms indicate the biphasic development cycle of *C. burnetii* and the differential regulation involved.

The Host Endocytic Pathway

The *C. burnetii* CCV is highly fusogenic and acquires cellular markers through heterotypic fusion events with the host endocytic pathway. Host endocytosis begins with the formation of vesicles through membrane fission events. These vesicles termed early endosomes contain endocytosed material such as membrane components, macromolecules, particles, and fluid solutes [18, 19] (Figure 1). The pH of the early endosome (~6.2) is achieved through the V-ATPase proton pump. Maturation of the

host early endosome to the late endosome with a pH of approximately 5.5 is mediated by GAPs (GTPase-activating proteins) and GEFs (guanine nucleotide exchange factors) that act on Rabs [20]. GTPases are critical membrane-associated proteins that function as regulators of vesicular trafficking pathways; they are commonly referred to as the Rab family. Rab5 is localized to early endosomes (EEs) through the GTP effector Rabaptin 5 that generates a positive feedback loop [21]. The conversion of Rab5 to Rab7 along with the loss of Early Endosome Antigen 1 (EEA1) drives the maturation of the EE to the late endosome (Figure 2). The late endosome is a multivesicular body that then fuses with lysosomes that have a pH of approximately 4.5 [20]. EEs have been shown to function as nutrient sensing organelles that also mediate the starvation response and regulates energy metabolism. In addition, autophagosomes are formed with the aid of Atg (autophagy related gene) proteins such as LC3 and LC3-II - its modified form. The autophagosomes then fuse with lysosomes until they are digested by lysosomal proteases to be used by the cell [22]. The motility of late autophagosomes and late endosomes on microtubules is mediated by Rab7, RILP, and ORP1L [18]. The host endocytic pathway concludes with the formation of mature acidic lysosomes containing the glycoprotein LAMP1. It is a common marker for lysosomes, however, LAMP-1 labeled vesicles are heterogeneous [23]. The mature CCV acquires these cellular markers and remains around pH ~5.2 [24].

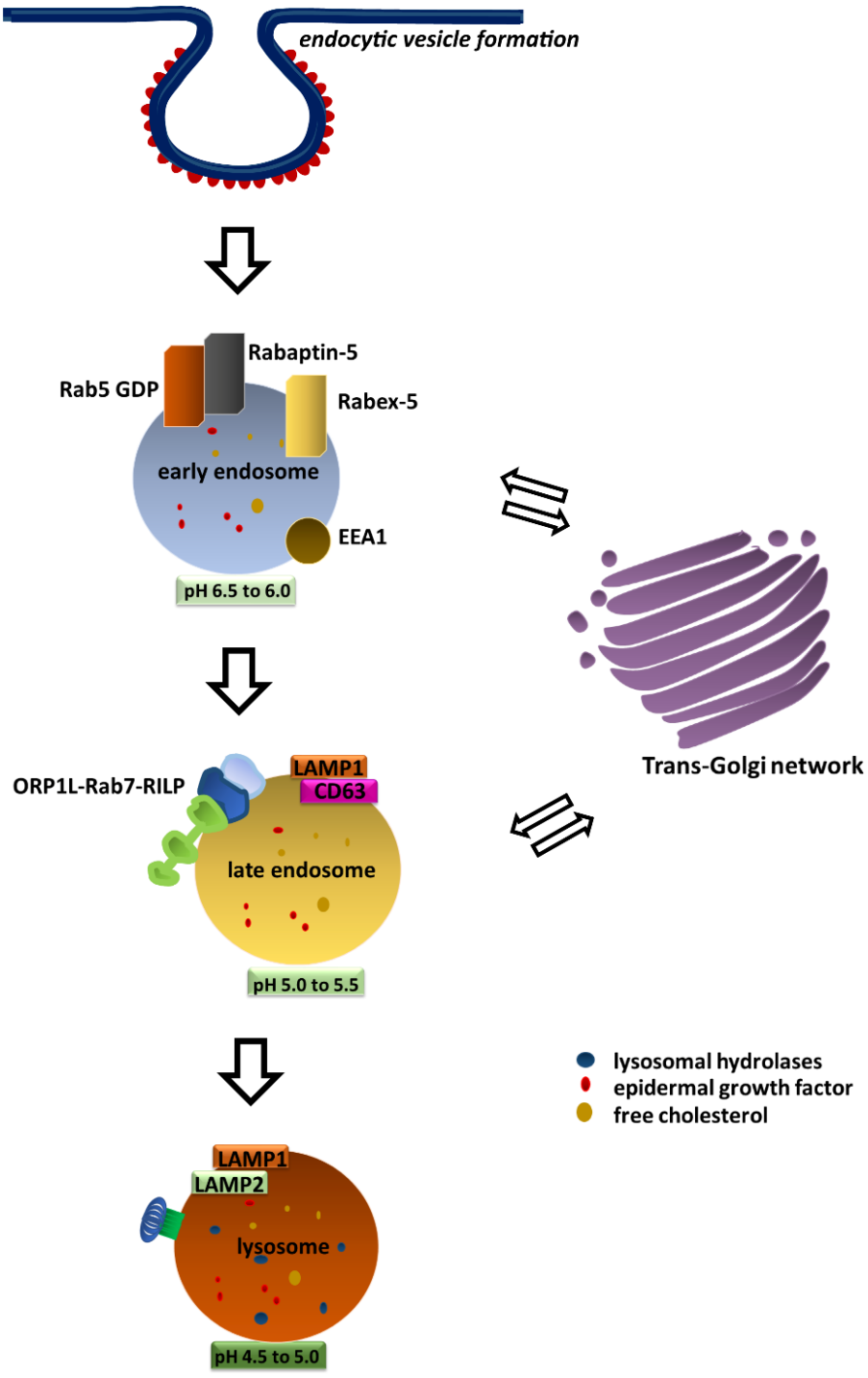

endocytic vesicle formation
Rabaptin-5
Rab5 GDP
Rabex-5
early endosome
EEA1
pH 6.5 to 6.0
Trans-Golgi network
ORP1L-Rab7-RILP
LAMP1
CD63
late endosome
pH 5.0 to 5.5
lysosomal hydrolases
epidermal growth factor
free cholesterol
LAMP1
LAMP2
lysosome
pH 4.5 to 5.0

Figure 1. The Host Endocytic Pathway. The endocytic pathway begins when membrane fission events produce vesicles termed early endosomes that accumulate cargo such as macromolecules and membrane components. The early endosomes mature into late endosomes by conversion on Rab5 to Rab7. Early endosomes containing Rab5 and late endosomes containing Rab7 mediate exchange and transport of compartments with the trans-Golgi network (TGN). These vesicle populations carry a specific subset of endocytosed cargo and remain around pH 6.0 to 5.5. They mature through homotypic fusion events and generate the classical lysosome containing lysosomal +++hydrolases and pH 4.5.

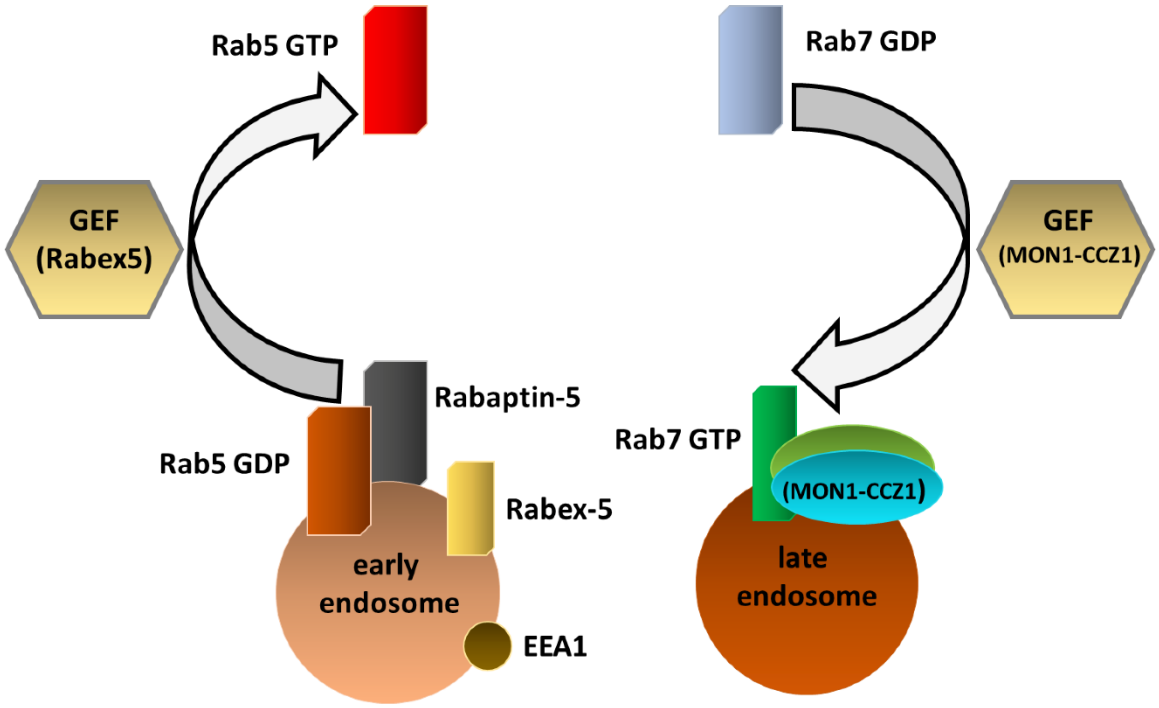

Rab5 GTP
GEF
(Rabex5)
Rab5 GDP
Rabaptin-5
Rabex-5
early
endosome
EEA1
Rab7 GDP
GEF
(MON1-CCZ1)
Rab7 GTP
(MON1-CCZ1)
late
endosome

Figure 2. Rab5 to Rab7 Conversion. Early endosomes contain the GTPase Rab5 that distinguishes it from the late endosomes. Rab5 is found on early endosomes forming a complex with Rabex-5, a GEF, and effector Rabaptin 5. The conversion of Rab5 to Rab7 is mediated by GAPs and GEFs that facilitate the GTP hydrolysis events. Rab5 is then replaced with Rab7 on late endosomes. Recruitment of the Rab7 GTPase occurs through the protein Mon1-Ccz1 that acts as a GEF and allows for the maturation of the early endosome to the late endosome.

C. burnetii Life Cycle

The *C. burnetii* life cycle within host cells is well characterized and it has been established that entry into a host cell is a phagocytic event and occurs through host cell receptors: CR3 (complement receptor-3), CD47 (integrin-associated protein) and $\alpha_v\beta_3$ integrin [10, 25]. Following internalization into the host cell, unlike most intracellular pathogens *Coxiella* actively fuses with the host endosomal cascade and does not avoid fusion with lysosomes (Figure 3). The nascent CCV fuses with the endosomal cascade and recruits Rab5, mannose receptors, and Rab7 approximately 40 minutes post-infection [26]. The CCV then begins to acidify and this is thought to occur by acquiring its lysosomal characteristics, acidic pH, and lysosomal membrane by fusion with host lysosomes. It acquires LAMP-1 (Lysosome-associated membrane glycoprotein-1) and other glycoproteins such as LAMP-2 and LAMP-3 (commonly referred to as CD63) [24, 26]. These fusion events with the host lysosomes have been reported to occur 2 to 6 hours post infection and results in the recruitment of lysosomal enzymes such as acid phosphatases, hydrolases, and cathepsin D [13, 24].

These events increase the metabolic activity of *Coxiella* and establishes an acidic pH within the CCV that upregulates expression of the Dot/Icm type IV secretion system (T4SS)[27]. The T4SS is a pilus-like structure that spans the inner and outer bacterial membranes forming a translocation pore in the CCV membrane through which *Coxiella* delivers approximately 130 effector proteins to manipulate host cell processes, initiate intracellular replication and promote CCV expansion [27, 28].

The phagosome maturation process continues while *Coxiella* actively fuses with the host endocytic pathway. This then results in the late phagosome that acquires endocytic SNARE proteins such as syntaxin-17 and Vamp7 that have been shown to mediate homotypic fusion events of *Coxiella* phagosomes [29]. Approximately 48 hours post infection the CCV begins to recruit autophagy proteins such as microtubule-associate protein 1A/1B light-chain 3 (LC3), p62, and Beclin through PV and mature autophagosome interactions [30, 31]. Although the mature CCV has been shown to be negative for the presence of EE markers such as EEA1, mutants of *Coxiella* proteins that

target the CCV membrane exhibit fatal defects in CCV formation and intracellular replication [32] [33]. These findings indicate that the both host and bacterial protein localization on the CCV membrane are required to maintain steady CCV biogenesis and intracellular growth.

The host cell remains viable despite the expansion of the CCV and this could be attributed to the anti-apoptotic activity and activation of pro-survival factors by *C. burnetii* [34]. The survival of infected cells may be critical for the spread and maintenance of both acute and chronic infections in mammals.

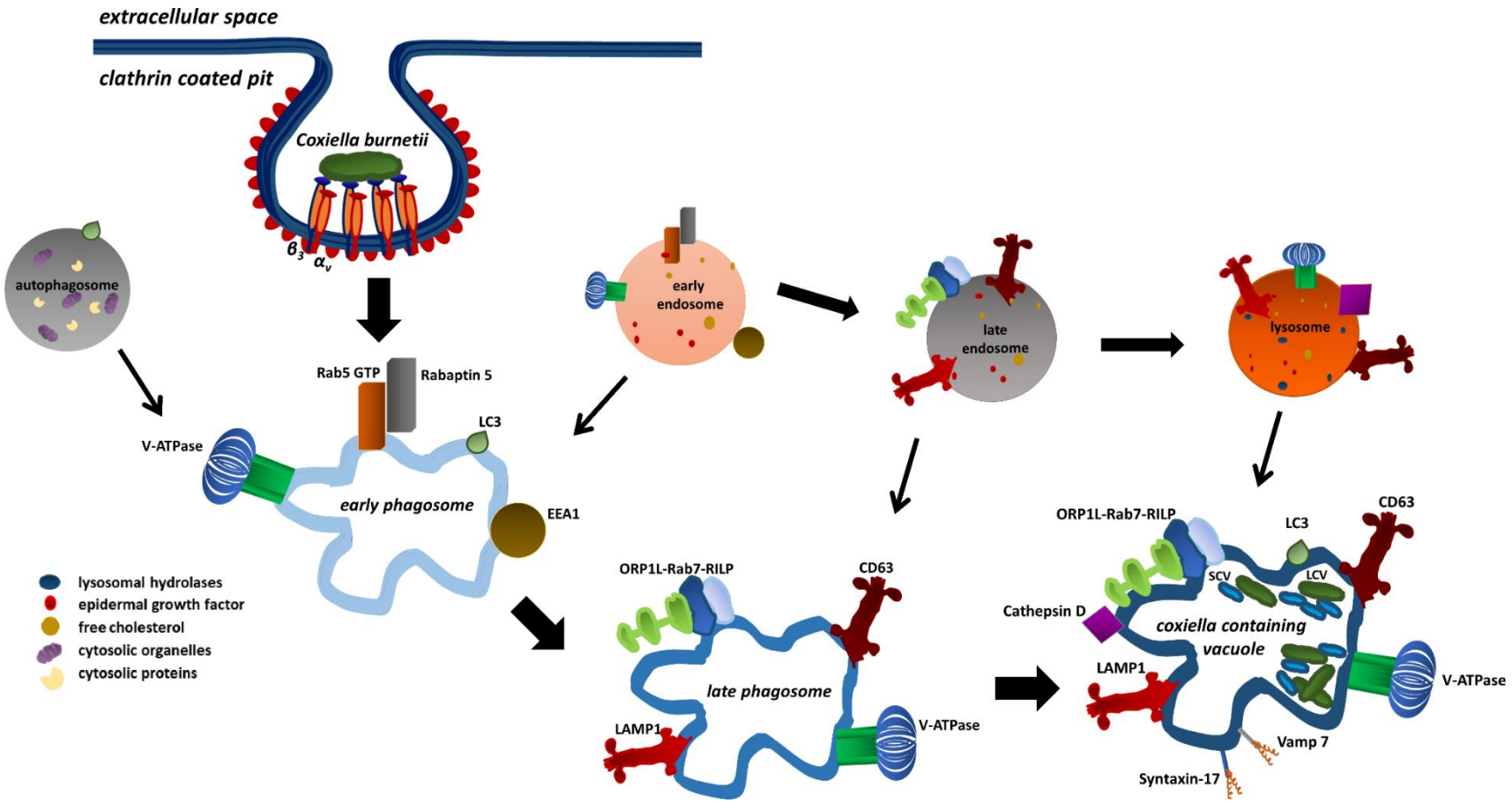

extracellular space
clathrin coated pit
Coxiella burnetii
β_3 α_v
autophagosome
early endosome
late endosome
lysosome
Rab5 GTP
Rabaptin 5
LC3
V-ATPase
early phagosome
EEA1
lysosomal hydrolases
epidermal growth factor
free cholesterol
cytosolic organelles
cytosolic proteins
ORP1L-Rab7-RILP
CD63
late phagosome
LAMP1
V-ATPase
ORP1L-Rab7-RILP
LC3
CD63
SCV
LCV
Cathepsin D
coxiella containing vacuole
LAMP1
V-ATPase
Vamp 7
Syntaxin-17

Figure 3. *Coxiella* Fusion with the Host Endocytic Pathway. *Coxiella burnetii* is endocytosed by binding to host adhesion receptor αvβ3 integrin. The early phagosome fuses matures by fusing with early endosomes and autophagosomes while acquiring cellular markers such as Rab5 and LC3. The early phagosome then matures with the more acidic vesicles such as late endosomes and acquires markers such as ORP1L, Rab7, RILP and CD63. The maturation process continues as the *Coxiella* phagosome fuses with host lysosomes. At this stage the phagosome matures into a phagolysosome where it acquires lysosome specific components such as hydrolases and the LAMP1**.**

Cholesterol and *Coxiella* Pathogenesis

Cholesterol has been implicated in important roles for host-pathogen interactions and has been recognized as a critical structure in pathogen-occupying vesicles [35]. Obligate intracellular pathogens such as *C. burnetii* are dependent on the host cell for substantial amounts of the growth requirements and therefore, they have evolved intricate mechanisms to obtain vital nutrients [36]. They can then manipulate the cholesterol trafficking pathways inside the host cells to acquire host nutrients and maintain the intracellular niche [37]. These processes are predicted to occur by hijacking cholesterol uptake, metabolism, and storage [36].

Coxiella has been shown to have a sterol rich CCV membrane decorated with lipid raft proteins flotillin-1 and flotillin-2 [38, 39]. The presence of lipid raft proteins and the mechanically strong structure of CCV membrane suggests that cholesterol may play a role in the CCV structural integrity [39]. Interestingly, the pathogen encodes a putative Δ24 sterol reductase homolog CBU1206; this enzyme is absent in other prokaryotes [40]. The mammalian homolog of the Δ24 sterol reductase is DHCR24, which is responsible for catalyzing the final step in cholesterol biosynthesis by reducing sterol double bonds [39]. Previous studies demonstrate that the *C. burnetii* CBU1206 is able to reduce the C24 double bond of the ergosterol precursor in a *Saccharomyces cerevisiae* model [40]. The ability of CBU1206 to modify the yeast sterol precursor indicates that it is an active enzyme that might serve a critical function in modifying sterols. Since *Coxiella* infects mammalian cells that maintain cholesterol as the central lipid for cellular lipid homeostasis, it is unlikely that CBU1206 is used for producing cholesterol [41]. It has been shown that high levels of cholesterol leads to over-acidification of the CCV, defective fusion with the host endocytic pathway, and bacterial death [42]. Predicted functions of CBU1206 include conversion of cholesterol precursors to novel sterols or modification of cholesterol to a sterol more beneficial to *C. burnetii*.

Research Goals

Building upon previous work indicating that increasing cholesterol concentrations are bacteriolytic to *C. burnetii.* We hypothesize that CCV cholesterol concentration is meticulously regulated through the *Coxiella* sterol reductase CBU1206. In order to better understand the mechanisms of CBU1206, the effects of altered lipid content on the CCV membrane and heterotypic fusion events; I observed the localization of host proteins by investigating a complete knockout mutant of the *Coxiella* Δ24 sterol reductase CBU1206 (*ΔCBU1206*).

Firstly, the effects of sterol deregulation on heterotypic fusion events with the *ΔCBU1206* CCV were investigated. The sterol deregulation effects were observed by scoring the localization of endolysosomal host proteins such as CD63, LAMP1, Rab7, ORP1L, RILP, and LC3 on the mutant CCV membrane as WT CCVs readily acquire proteins during infection. These fusion events are essential for maintaining an acidic and expanding vacuole that leads to disease. Our aim in investigating the localization of these cellular markers to the CCV membrane is to gain a deeper understanding of the relevance of these host proteins to the developing CCV. I expect to see reduced or heavily increased localization of both late endosome and lysosome markers due to the mutant having to compensate for the lack of a sterol reductase enzyme through currently unknown mechanisms.

Secondly, the functional capacity of the Δ24 sterol reductase mutant Type 4 Secretion System (T4SS) will be determined. The secretion system is a critical virulence factor that facilitates the secretion of approximately 130 effector proteins. These effectors regulate numerous aspects of the host cell to manipulate the host defense in response to *Coxiella* infection. These modulations of the host cell allow for a replication-permissive vacuole that results in disease progression. The extent of effector secretion through the T4SS will be measured through a CyaA Reporter Assay that measures secretion efficacy through adenosine triphosphate (ATP) conversion to Cyclic AMP (cAMP). I expect to see a moderately functional T4SS for the mutant indicating that the phenotypes observed with the mutant CCV cannot be solely due to lack of effector

proteins but also because of aberrations in sterol regulation. Overall, these two aims allow us to further understand the function and importance of the *Coxiella* sterol reductases while illuminating potential therapeutic targets.

MATERIALS AND METHODS

Bacterial Strains

C. burnetii NMII Phase II (NMII, RSA439), *C. burnetii ΔCBU1206* (NMII; clone 41), *ΔdotA* mutant (2) and mCherry expressing strains were grown for 8 days in 1X ACCM-D media and washed twice with 1X PBS as described in (Omsland et al., 2009). For all experiments counting the presence of host proteins on the *C. burnetii* CCV, all strains were grown for 8 days in ACCM-D (nutritionally defined medium with known amino acid concentrations) (Sandoz et al., 2016) and washed twice with 1X PBS as described in (Omsland et al., 2009). The multiplicity of infection (MOI) was optimized for the (WT) *C. burnetii* NMII Phase II, and *ΔCBU1206* for ~1 internalized bacterium per cell.

Mammalian Cell Culture

HeLa cells and Murine alveolar macrophages (MH-S cells) were maintained in RPMI (Roswell Park Memorial Institute medium) 1640 medium (Corning, New York, NY, USA) that contained 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA), and glutamine (Gibco, Paisley, Scotland, UK) 37°C and 5% CO_2.

Filipin Staining for Sterol Quantification

On a 6 well-plate 5 x 10^4 cells per well of HeLa cells were plated and infected the following day. *C. burnetii* NMII Phase II and *ΔCBU1206* were used to infect cells in 500 uL of RPMI 1640 medium at an optimal MOI. At 3 dpi cells were incubated with 5uM of U18666A (Fisher Scientific, Hanover Park, IL, US) for 4 hours at 37°C. These cells were then washed and fixed with 2.5% paraformaldehyde (PFA) (Alfa Aesar, Haverhill, MA, USA) for 15 mins and incubated with filipin at (1:100) (NC9384284; Fisher Scientific, Hanover Park, IL, US) for 1 hour in the dark. The cells were washed and mounted on slides with ProLong-Gold (Invitrogen).

Transfection with GFP Plasmids

5 x 10^4 cells per well of HeLa cells were seeded in a 24 plate in RPMI 1640 medium. They were transfected with pEGFP plasmids containing Fugene 6 (Promega, Madison, WI, USA), according to manufacturer's protocol. pEGFP plasmids encoding full-

length ORP1L, Rab7, RILP, and LC3 were transfected. Approximately 48 hrs post-transfection, the HeLa cells were infected with *C. burnetii* in 0.25 mL of 10% RPMI and incubated for 2 hours at 37°C, 5% CO_2. The cells were then washed extensively with 1X PBS and incubated in 10% RPMI. At 0, 4, 12, 24, 48 and 72 hours post infection, the coverslips were fixed with 2.5% PFA for 15 minutes. The cells were then blocked and permeabilized for 20 minutes with 0.1% saponin and 1% bovine serum albumin (BSA).

***C. burnetii* Recruitment of Host Proteins by Immunofluorescence Assay**

HeLa cells were plated at (2 x 10^5 cells per well of a 6-well plate for 0 and 4 hr) and (5 x 10^4 cells per well of a 24 plate for 12, 24, 48 and 72 hours) and allowed to adhere overnight. For immunofluorescent labeling, HeLa cells were infected with mCherry *C. burnetii* NMII Phase II (WT) or mCherry *ΔCBU1206*, washed extensively with PBS, and incubated in 10% RPMI. At the indicated times post infection, infected cells were fixed with 2.5% PFA for 15 minutes. The cells were incubated with monoclonal antibodies mouse anti-CD63 (1:1000) (556019; BD Biosciences, San Jose, CA, USA), anti-LAMP1(1:1000) (ab24170; Abcam, Cambridge, MA, USA) and guinea pig anti-*Coxiella* serum (Coleman et al., 2007) for 1 h in 0.1% saponin BSA-PBS. The coverslips were then incubated with AlexFluor secondary antibodies (Invitrogen, Carlsbad, CA, USA) in 0.1% saponin BSA-PBS. The coverslips were mounted using ProLong-Gold plus 4',6'-diamidino-2-phenylindole DAPI (Invitrogen) and visualized.

Microscopy

Immunofluorescent images were obtained using a Leica inverted microscope DMI6000B. Images were obtained using the a (63 x oil immersion objective) and at least 20 CCVs were visualized for each condition during three biological replicate experiments. The CCVs were scored for the presence or absence of each of these host proteins (CD63, LAMP1, ORP1L, Rab7, RILP and LC3). All images were analyzed using the FIJI ImageJ software (written by Wayne Rasband at the US National Institutes of Health).

CyaA Translocation Assay

C. burnetii cultures grown in ACCM- 2 was spun down at 16000xg for 15 min and suspended in 10% glycerol. Ten micrograms of PJB-CAT-CyaA or PJB-CAT-CyaA-CvpA DNA was mixed with 50 uL of *C. burnetii* in a cold electroporation cuvette. The bacteria were electroporated at 1.8 kv, 500 ohms, at 25 microF and suspended in 950 µL of pre-cooled RPMI prior to culturing in ACCM-2. The WT cultures were then treated with 3 µg/ml of Chloramphenicol while the *ΔCBU1206* cultures received 3 µg/ml of Chloramphenicol and 3.5 µg/ml of Kanamycin. Chloramphenicol was used as a gene to confer resistance following transformation while Kanamycin was used to ensure *Coxiella* mutant status.

Six strains were generated where WT, *ΔCBU1206*, and *dotA C. burnetii* were transformed with either pJB-CAT:CyaA:CvpA or pJB-CAT:CyaA only. The expression of CyaA was verified by Western blot using a Cya-3D1 primary antibody (Santa Cruz, WB = 1:500) and a goat-anti-mouse HRP antibody secondary antibody (Abcam, WB = 1:2,500).

Fc-γ-receptor HeLa cells were plated (5 x 10^4 per well) on a 24 well plate and incubated in 10% RPMI (Invitrogen) overnight. The cells were over-infected with *C. burnetii* NMII Phase II (WT), *ΔCBU1206* or *ΔdotA* strains containing pJBCAT:CyaA:CvpA or the vector alone. The cells were incubated in 10% RPMI for 48 h. The cells were then harvested and re-suspended in diluted assay buffer provided with the Amersham cAMP Biotrak Enzymeimmunoassay kit. The assay was performed according to protocol RPN2251. Standards were prepared according to protocol from 0 to 6400 fmol to generate a standard curve. The cAMP concentration of each sample was determined by plotting the pJB-CAT:CyaA:CvpA strains over the pJB-CAT:CyaA only strains.

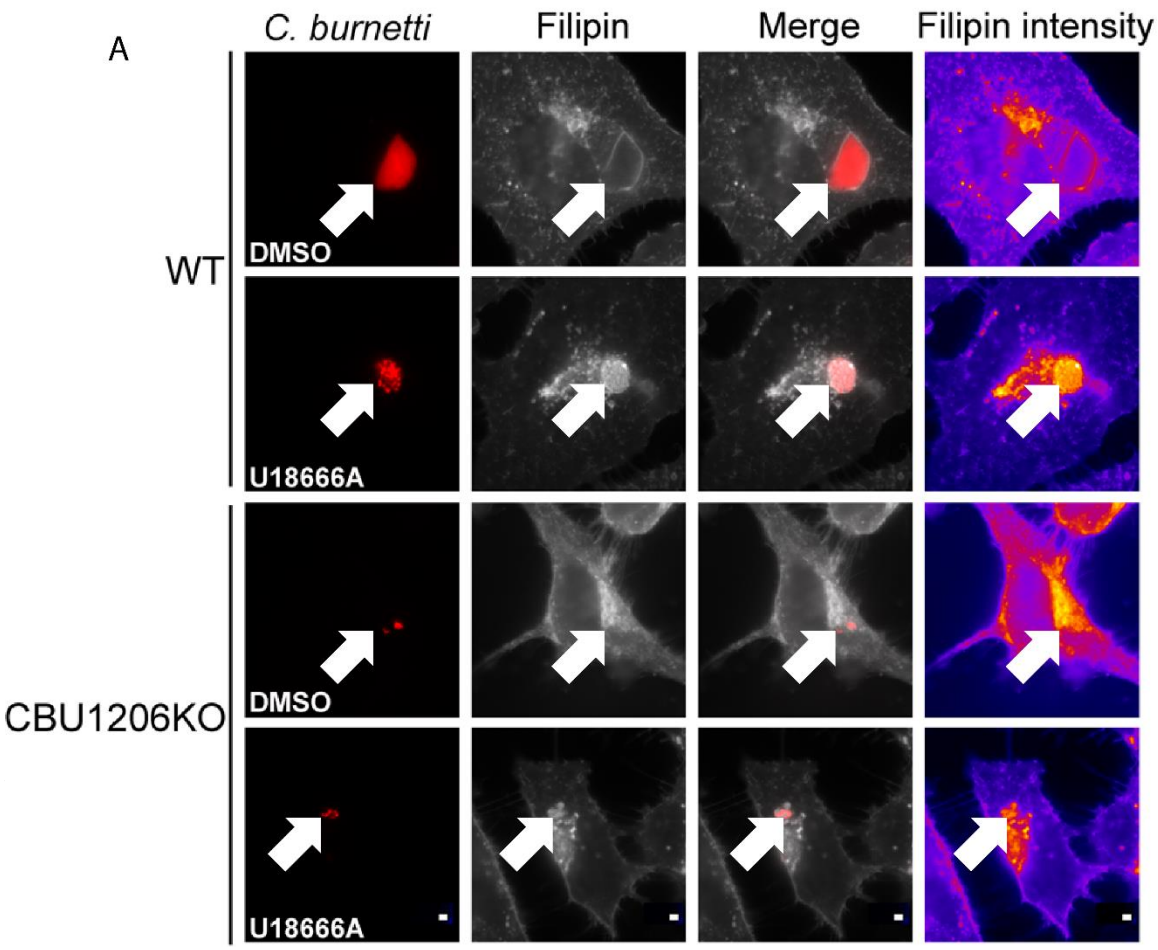
Statistical Methods

All statistical analyses were performed using the Prism software by GraphPad (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Increased Sterol Accumulation on the *ΔCBU1206* CCV Membrane

Previously the lab hypothesized that CBU1206 may modify cholesterol in the CCV to generate sterol species beneficial for survival. Therefore, the lab generated a *ΔCBU1206* mutant utilizing SacB targeted deletion. The potential accumulation of cholesterol on the CCV membrane due to lack of CBU1206 was tested using the *ΔCBU1206* and the use of fillipin. This compound is a four isomeric polyene that binds to sterols that are not esterified such as cholesterol. It binds to cholesterol in membranes and forms aggregates at the ultrastructural level that can be observed with UV excitation around 360 nm and emission around 480 nm [43]. To visualize the cholesterol content on the membrane of WT and mutant infected cells were labeled with filipin. Since non-esterified sterols could be numerous in structure and states of esterification, I used a positive control: U18666A. It has been shown to inhibit lipid transport resulting in an accumulation of cholesterol in lysosomal structures and therefore; it was used to induce cholesterol accumulation on the CCV membrane [44]. The cells were incubated with U18666A prior to labeling with fillipin. To confirm accumulation of cholesterol on the mutant infected and U18666A treated cells, fillipin intensity was visualized and quantified using FIJI ImageJ software (Figure 4 A, B). The higher intensity of filipin in the mutant CCV membrane indicated increased sterol accumulation that is likely cholesterol.


A
C. burnetti
Filipin
Merge
Filipin intensity
WT
DMSO
U18666A
CBU1206KO
DMSO
U18666A

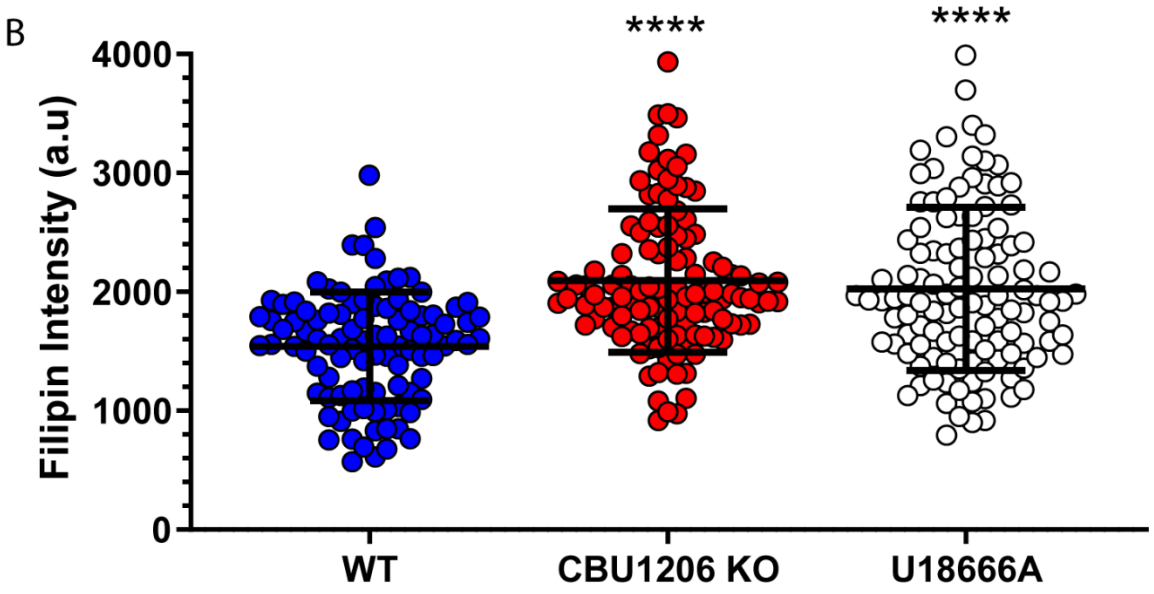

B
Filipin Intensity (a.u)
4000
3000
2000
1000
0
****
****
WT
CBU1206 KO
U18666A

Figure 4. Increased Sterol Accumulation on the *ΔCBU1206* CCV Membrane. HeLa cells were treated with U18666A or left untreated, fixed, and labeled with filipin at 3 days post infection (A). Sterol accumulation is indicated by increased filipin intensity that can be observed in U18666A treated cells relative to DMSO controls. *ΔCBU1206* infected cells also display increased filipin intensity relative to WT infected cells, particularly the DMSO controls where no cholesterol accumulation should occur. (B) Quantification of filipin intensity at 3 days post infection using FIJI ImageJ software. The individual CCV measurements from four separate experiments are shown. At least 20 CCVs were used in each experiment. Error bars indicate standard deviation from the mean. Statistical analyses used were Ordinary One-Way Anova with a Dunnett's Multiple Comparison Test. **** = $p < 0.0005$. Arrows point to CCVs. Scale bar = 1.82 µm.

Method Used to Determine Protein Localization on the CCV

The CCV acquires cellular markers such as CD63, Rab7, LAMP1, LC3, and SNARE proteins such as Vamp2, Vamp7, Vamp8 and Vti1b through fusion with the endocytic pathway and autophagosomes [45]. The endocytic pathway is composed of early endosomes, lysosomes, endolysosomes, and lysosomes that are continuously being modified or degraded. EEs undergo maturation and become acidified in the endocytic pathway. These mature into LEs that form larger bodies that fuse with lysosomes and organelles transitioning between endosomes and lysosomes. Collectively the EEs, LEs, endolysosomes, and lysosomes form a dynamic continuum that is continuously undergoing maturation and transformation events.

The CCV membrane has been shown to be altered in response to decreasing and unstable CD63 and LAMP1 levels suggesting an essential role for endosomes and lysosomes in CCV development [46]. Through immunofluorescent microscopy visual confirmation of host endosomal/lysosomal marker localization to the CCV was scored positive or negative for localization (Figure 5A, arrow). The absence of apparent localization indicated by the lack of a visible outline by the marker was scored as negative for localization (Figure 5B, arrow).

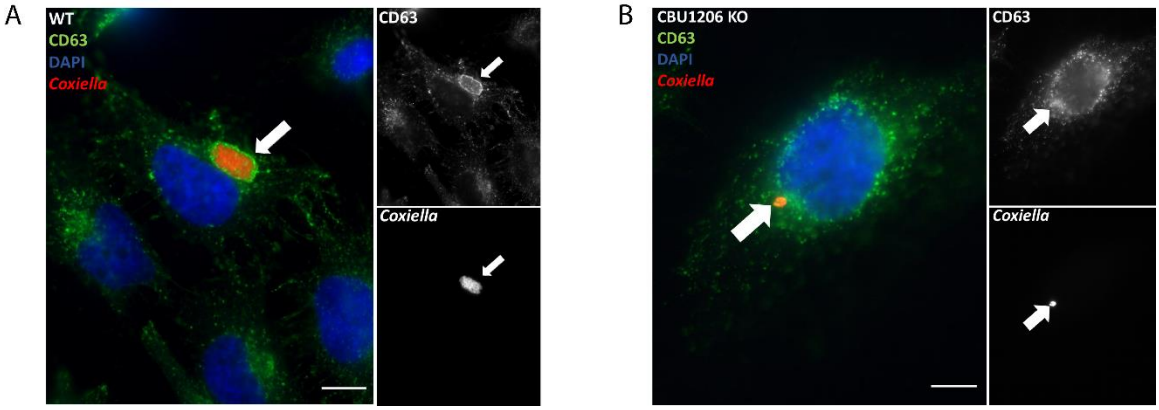

A
WT
CD63
DAPI
Coxiella
CD63
Coxiella
B
CBU1206 KO
CD63
DAPI
Coxiella
CD63
Coxiella

Figure 5. Scoring Localization of CCV Markers on Infected CCVs. CD63 localizes to the CCV membrane in HeLa cells infected with (WT) mCherry *C. burnetii* NMII Phase II but does not fully localize in mCherry *ΔCBU1206* infected cells at 3 dpi. Immunofluorescent microscopy images of WT and mutant HeLa cells stained with monoclonal anti-CD63. In the HeLa cell infected with WT, CD63 was seen localizing to the CCV membrane (A, arrow) and was scored as a positive PV (A). Host cell infected with the mutant displayed no localization of CD63 on CCV membrane (B, arrow) and was scored as negative (B). At least 20 CCVs were scored as positive or negative for the presence of CD63. Arrows point to CCVs. Scale bars = 2mm

CD63 is Present on a Decreased Number of Mutant CCVs

Lysosomes are one of the more unique vesicle populations in the endosomal maturation cascade and with an acidic pH of ~4.5. CD63 was one of the host proteins that were used as a marker for the fusion of lysosomes with the CCV. Primarily associated with the membranes of intracellular vesicles, CD63 is a ubiquitous protein in humans and is a member of the transmembrane 4 superfamily that is commonly referred to as the tetraspanin family. CD63 is associated with cell-surface proteins that play critical roles in cell development and growth. Although CD63 is a member of the LAMP family that is present on lysosomes it is also found on late endosomes [47]. It has been shown to colocalize with cation transporter 2 (hoCT2) and small GTPase Rab4 that are critical in recycling of the sorting endosomes to the plasma membrane on the cell surface [48] [49]. In *Burkholderia thailandensis* tetraspanins such as CD9, CD63, and CD81 were shown to be involved in the regulation of multinucleated giant cell formation (MNGC) formation that could be induced by bacterial pathogenicity [50]. Recently it was demonstrated that $CD63^+$ vesicles are not successfully eliminated from the *Coxiella* CCV without lysosomal hydrolases leading to an excess of intraluminal $CD63^+$ vesicles. The excess of intraluminal vesicles resulted in an altered CCV membrane suggesting proper distribution of vesicles within the CCV is essential for development [46]. To understand the distribution and fusion of CD63 vesicles with the *ΔCBU1206* infected CCVs, a time course of infection was conducted. The cells were visualized using immunofluorescent microscopy and scored by eye (Figure 6). Approximately 90% of the total WT CCVs displayed positive localization of CD63 beginning at 12 hours post infection while only about 30% of total mutant CCVS displayed positive localization. At 24, 48, and 72 hours post infection WT infected CCVS displayed >90% positive localization of CD63 while only about 50% of the total mutant CCVs acquired CD63 across all three timepoints. This data indicates that CD63 is acquired by a substantial group of WT CCV population beginning at 24 hpi. This is in stark contrast with the mutant CCVS where 70% of mutant CCVs failed to acquire CD63 or does not fuse with $CD63^+$ vesicles until 48 hours after infection. The time points 24, 48, and 72 hpi maybe critical stages in CCV development

as all WT CCVs are positive for the presence of CD63. The lack of CD63 on the mutant CCV membranes may indicate delayed vesicle fusion or a defective membrane that does not allow for proper fusion with late endosome/lysosome vesicles.

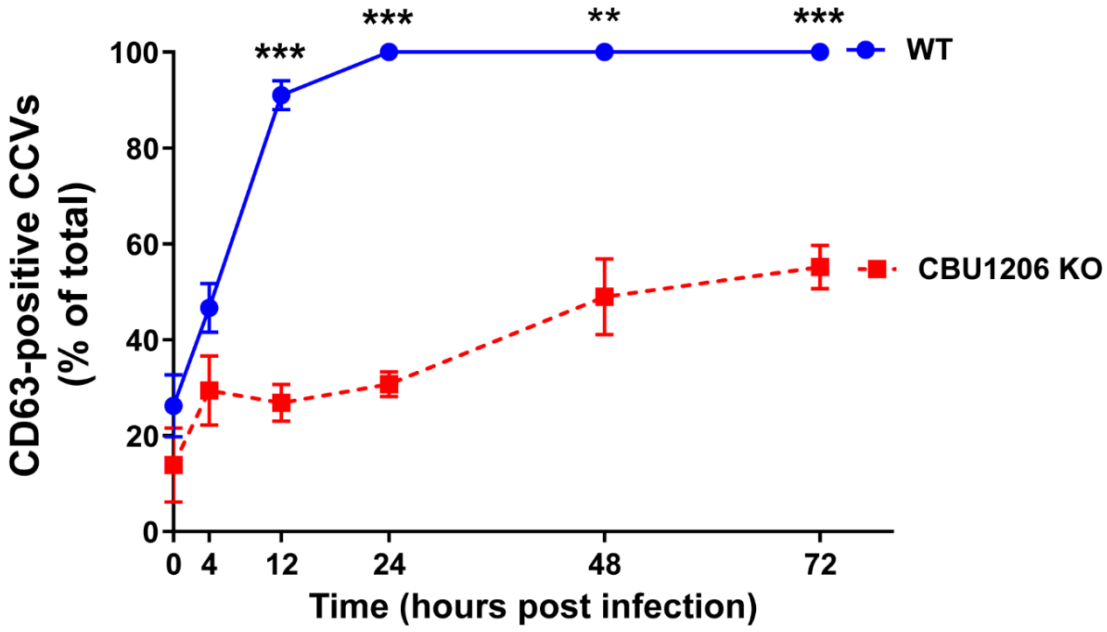

***
***
**
***
100
80
60
40
20
0
CD63-positive CCVs
(% of total)
WT
CBU1206 KO
0
4
12
24
48
72
Time (hours post infection)

Figure 6. CD63 is Present on a Decreased Number of Mutant CCVs. CD63 localizes to the CCV membrane of HeLa cells infected with (WT) mCherry *C. burnetii* NMII Phase II. At 24, 48, and 72 hpi >90% of WT CCVs were positive for CD63. At 24 hpi, approximately 30% of mutant CCVs were positive. At 48 and 72 hpi about 50% of the mutant CCVs were CD63 positive. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$

LAMP1 is Present on Mutant CCVs

LAMP1 was the primary host protein used as a marker for the fusion of lysosomes with the CCV. Lysosomal Associated Membrane Protein 1 or LAMP1 is a protein in the family of membrane glycoproteins. LAMP1 is a heavily *N*-glycoslyated integral protein on the lysosomal membrane that is trafficked from the trans-Golgi network to the lysosomes [51]. It has been heavily associated with maintaining the lysosomal pH, integrity and catabolism. Most intracellular pathogens such as *Mycobacterium*, *Salmonella*, *Legionella* and *Toxoplasma* actively manipulate the host endocytic pathway to avoid fusion with lysosomes, however, *Salmonella*-containing phagosomes have been shown to acquire LAMP1 [52] [53]. *S. typhimurium* has been shown to recruit LAMP1 by fusing with Golgi-derived vesicles through a SipC-Syntaxin6-mediated event surpassing the need to fuse with lysosomes [53]. *Neisseria* has been shown to promote the degradation of LAMP1 through the *Neisseria* type 2 IgA1 protease allowing for its intracellular growth and pathogenesis [54]. A group looking at internalization routes of *C. burnetii* and the effects of vesicular trafficking on that process concluded that lack of LAMP1 and LAMP2 results in increased fusion with the endosomes and lysosomes but does not result in PV enlargement [55]. Another study claimed somewhat contradictory findings where fusion events of the CCV membranes were decreased due to lower levels of LAMP1 resulting in smaller CCVs [46]. Regardless of conclusions regarding fusion events both studies conclude that LAMPs play an essential role in the expansion of the CCV. This was further supported by CCV lysates that were significantly enriched for the presence of LAMP1 and LAMP2 relative to whole cell lysates possibly indicating crucial roles for the CCV expansion and maintenance [56]. While the effect of LAMP1 has been somewhat characterized in the WT CCV, I hoped to understand CCV and LAMP1 interactions through the *ΔCBU1206* infected CCV. Infected cells were visualized using immunofluorescent microscopy and scored by eye (Figure 7). Unlike the delayed acquiring displayed with other markers, the mutant displayed a different pattern of localization that is similar to WT. At 24 hpi almost all (>95%) the WT CCVs scored positive for the presence of LAMP1 and remained positive through 48 and

72 hpi. Around 70% of the total mutant CCVs also scored positive for the presence of LAMP1 on the CCV membrane and 100% of the mutant CCVs were positive for LAMP1 at 72 hpi. The mutant CCVs become increasingly positive for the presence of LAMP1 unlike with other CCV markers but still displays delayed recruitment relative to WT. The mature CCV membrane has been shown to acquire all the lysosomal glycoproteins LAMP-1, LAMP-2 and LAMP-3 while decreased levels of LAMP1 causes smaller CCVs [13, 55]. These findings might implicate a critical role of LAMP1 in CCV development and formation from early infection to late infection both in WT and mutant CCVs.

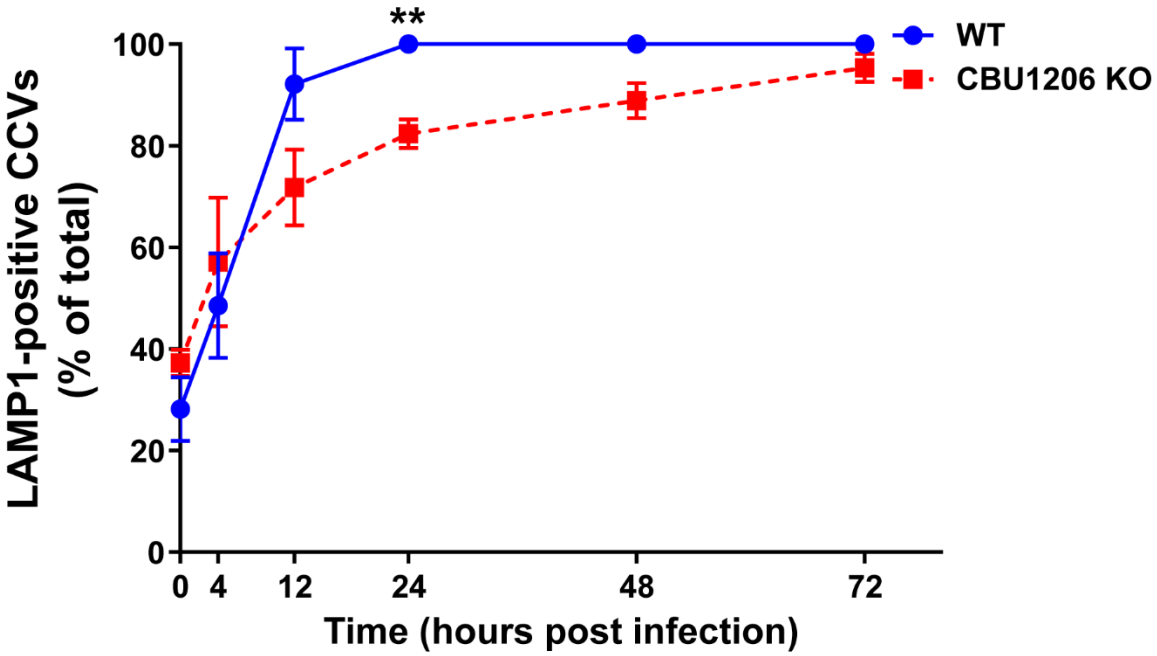

**
WT
CBU1206 KO
LAMP1-positive CCVs
(% of total)
100
80
60
40
20
0
0
4
12
24
48
72
Time (hours post infection)

Figure 7. LAMP1 is Present on Mutant CCVs. LAMP1 localizes to the CCV membrane of HeLa cells infected with (WT) mCherry *C. burnetii* NMII Phase II and mCherry *ΔCBU1206* infected cells. At 24, 48 and 72 hpi >95% of the WT CCVs and >60% of the mutant CCVs were positive for LAMP1. At 72 hpi 100% of both WT and mutant CCVs were positive for LAMP1. At least 20 CCVs were scored as positive or negative for the presence of LAMP1. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

Rab7 is Present on a Decreased Number of Mutant CCVs

Late endosomes contain distinct late endocytic cargo and a pH of ~5.0. They deliver these unique components to the CCV post fusion. In order to better understand late endosome recruitment to the CCV in the absence of CBU1206, I used Rab7, ORP1L, and RILP as late endosomal markers. The RAB7A gene encodes the Ras-related protein Rab-7a (Rab7) that is a small GTPase of the Rab family. Rab7 can be found co-localizing with ORP1L on late endosomes and lysosomes [57]. Rab7 and its effector (Rab-interacting lysosomal protein) RILP primarily localizes to late endosomes and directs the transport of early endosomes to the Trans Golgi Network (TGN) while playing a crucial role in endosomal trafficking [58]. Its role in regulating endosomal sorting, lysosomal biogenesis, and phagocytosis makes it relevant in the context of intracellular pathogen infection [59]. Rab7 and its partner GTPases have been characterized as key regulators in the phagosome maturation process of several intracellular pathogens. *Mycobacterium tuberculosis* phagosome maturation is arrested in early stages through selective exclusion of Rab7 effectively blocking fusion with late endosomes. This allows the *Mycobacterium*-containing phagosomes to escape degradation and thrive in its intracellular niche [60]. *Salmonella enterica* resides in *Salmonella*-containing vacuoles that have been shown to lose integrity when Rab7 is overexpressed in cells [61]. CCV fusion with the late endosomes requires Rab7 and the CCV has been shown to have substantial labeling of Rab7 at 48 hpi [62]. Expression of dominant-negative Rab7 mutant forms during infection has altered both CCV formation and intracellular replication [62] [63]. Considering the wide array of functions Rab7 has from endosomal sorting to phagocytosis, it was important to investigate mutant recruitment of Rab7 containing vesicles. Transfected cells were infected, visualized using immunofluorescent microscopy, and scored by eye (Figure 8). Approximately 50% of the total WT CCVs counted were positive for the presence of Rab7 and about 90% of CCVs were positive at 48 hpi. Although all the WT CCVs were positive for the presence of Rab7 on the membrane only about 30% of mutant CCVs were positive for Rab7. Approximately 70% of the mutant CCVs fail to acquire Rab7 even at 72 hpi when the CCV is expected to be

stabilized. Lack of Rab7 on the mutant CCVs was observed across all time points relative to WT and may indicate that the mutant is unable to properly regulate late endosome and lysosome vesicle fusion events. Although, Rab7 is only slowly acquired relative to lysosomal markers (LAMP1 and CD63) it has been shown to affect CCV formation and replication, therefore, Rab7 positive vesicle fusion events maybe more minutely regulated by *Coxiella*.

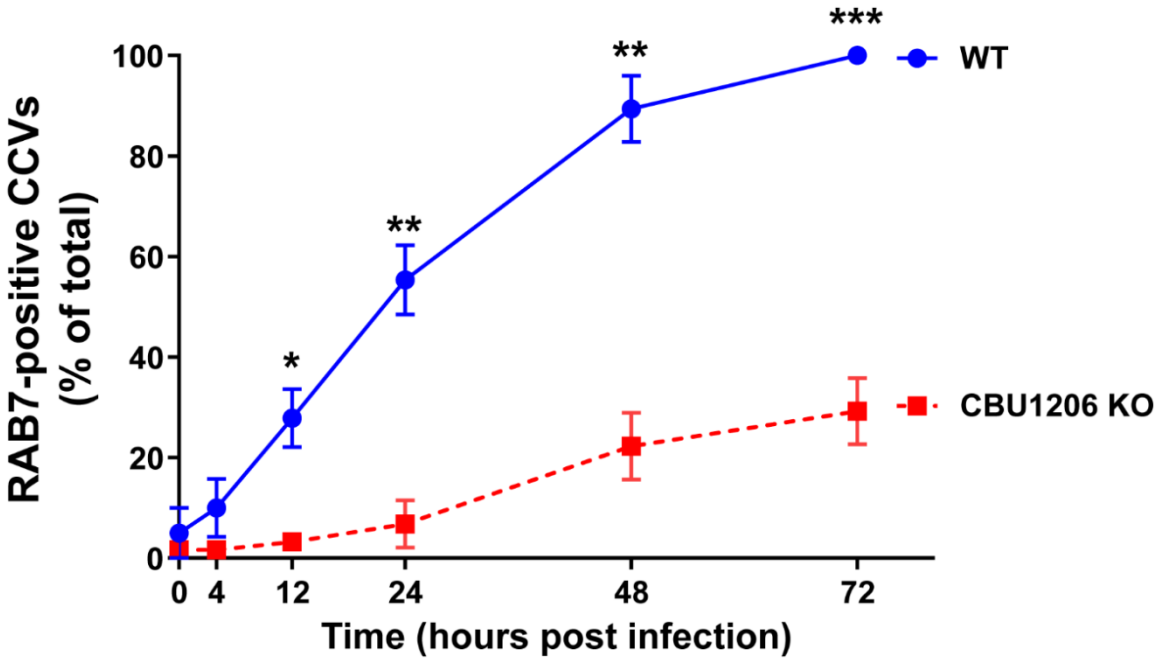

RAB7-positive CCVs
(% of total)
100
80
60
40
20
0
0
4
12
24
48
72
Time (hours post infection)
*
**
**
**
***
WT
CBU1206 KO

Figure 8. Rab7 is Present on a Decreased Number of Mutant CCVs. Rab7 localizes to the CCV membrane of HeLa cells infected with (WT) mCherry *C. burnetii* NMII Phase II steadily over 4, 12, 24, 48, and 72 hpi. CCVs infected with mCherry *ΔCBU1206* did not readily acquire Rab7. At 48 and 72 hpi, >90% of WT CCVs and <50% of mutant CCVs were positive for Rab7. At least 20 CCVs were scored as positive or negative for the presence of Rab7. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

ORP1L is Present on a Decreased Number of Mutant CCVs

Oxysterol-binding protein-related protein 1 or ORP1L is a member of the human oxysterol-binding protein (OSBP) family. ORP1L is primarily found on late endosomes and lysosomes [57]. It modulates the cholesterol levels on late endosomes through conformational changes dictated by high and low levels of cholesterol. When cholesterol levels are high, ORP1L binds to cholesterol through its C-terminal oxysterol-binding protein (OSBP)-related domain (ORD) and adopts a confirmation to enable the N-terminal ankyrin repeat domain (ARD) to interact with the Rab7-RILP complex positioning late endosomes [64]. When cholesterol levels are low, cholesterol does not bind the ORD domain exposing the FFAT motif adjacent to it. The FFAT motif is detected by the endoplasmic reticulum protein (ER) VAP forming ER and late endosome membrane contact sites [65]. Previous work from the lab suggests that ORP1L localizes to the WT CCV in a Dot/Icm type IV secretion system (T4SS) dependent manner. ORP1L binds the CCV membrane proteins via N-terminal ankyrin repeats and is involved in CCV and ER interactions. Infection of cells depleted of ORP1L using siRNA resulted in smaller PVs at 6 days post infection. Furthermore, the recruitment of RILP and Rab7 to the CCVs in both normal and ORP1L-depleted cells indicated that diminished CCV size was not due ORP1L interacting partners [45]. ORP1L has been shown to have an essential role in the CCV size and CCV interactions with the ER, therefore; it is likely that ORP1L has a role in altering or maintaining the CCV membrane. Considering its close association to CCV membrane function, ORP1L localization was investigated in the context of *ΔCBU1206*. ORP1L localization to WT and mutant was observed. Infected cells were visualized using immunofluorescent microscopy and scored by eye (Figure 9). Corresponding with previous observations in the lab, ORP1L was rapidly acquired by the WT CCVs beginning 12 hpi with >80% of CCVs positive for ORP1L at 24 hpi [45]. More than 95% of WT CCVs acquired ORP1L at 48 hpi and all CCVs were positive at 72 hpi. Interestingly, the mutant CCVs also display and increase of ORP1L recruitment after 12 hpi but was not comparable to the WT increase. Only >15% of mutant CCVs were positive for the recruitment of ORP1L while >75% of WT showed recruitment of ORP1L. Although the

number of ORP1L-positive CCVs increased over time, at 72 hpi less than 60% of total CCVS were positive for the presence of ORP1L on the CCV membrane. Considering the role of ORP1L in CCV membrane function, delayed ORP1L recruitment maybe indicative of delayed CCV development and CCV interactions with the ER.

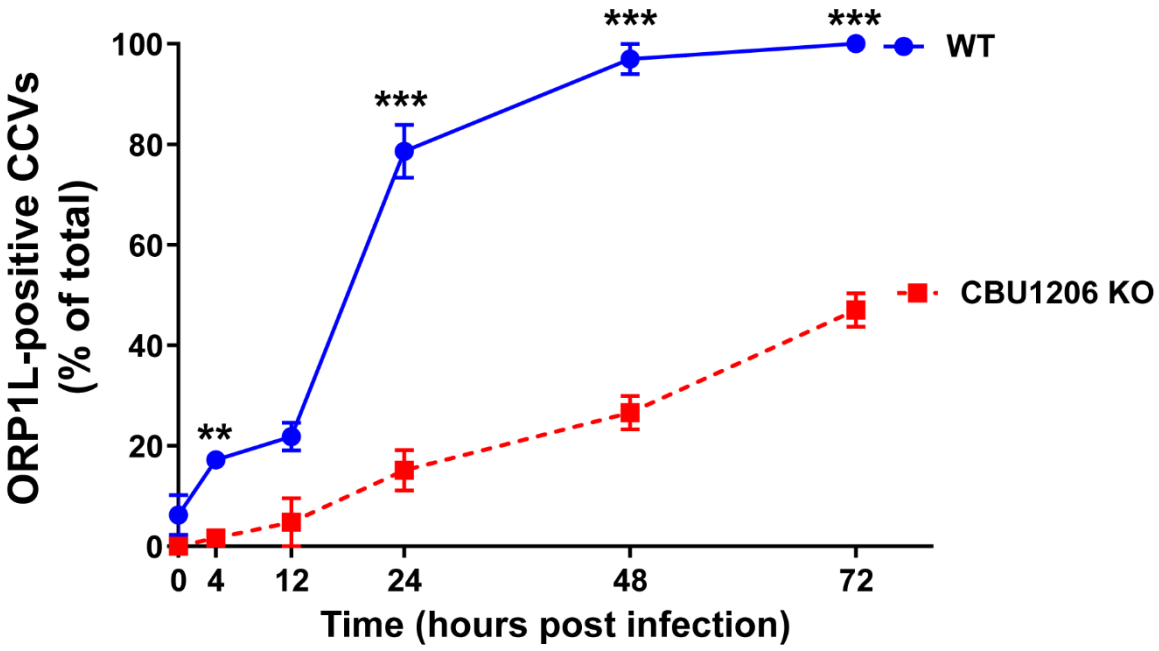

ORP1L-positive CCVs
(% of total)
100
80
60
40
20
0
**
***
***
***
0
4
12
24
48
72
Time (hours post infection)
WT
CBU1206 KO

Figure 9. ORP1L is Present on a Decreased Number of Mutant CCVs. ORP1L localizes to the CCV membrane of HeLa cells infected with (WT) mCherry rapidly following 12 hpi. CCVs infected with mCherry *ΔCBU1206* acquired ORP1L in a delayed manner relative to WT. At 24 hpi, >75% of WT CCVs and <30% of mutant CCVs were positive for ORP1L. At 72 hpi, 100% of the WT CCVs and approximately 50% of the mutant CCVs acquired ORP1L. At least 20 CCVs were scored as positive or negative for the presence of ORP1L. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

RILP is Present on a Decreased Number of Mutant CCVs

The 45 kDa protein binding to Rab7 at its C-terminus is the Rab-interacting lysosomal protein (RILP) [66]. It localizes to late endosomes and lysosomes acting as a downstream effector of Rab7 controlling the transport of endocytic compartments. RILP interacts with the Vps22 and Vsp36 subunits of the endosomal sorting complex (ESCRT-II) that are important for the biogenesis multivesicular endosomes (MVEs) that are the equivalent of late endosomes [67]. More relevant in the context of this study is the binding of the RILP in the ORP1L-Rab7-RILP tripartite complex that regulates late endosome positioning [64]. The complex is formed when RILP binds to the switch regions of Rab7 to arrest it in the active state. This is followed by the ORP1L interactions with the ER VAP proteins and binding to the Rab7-RILP complex thus forming the tripartite complex [68]. Further, recruitment of the dynein-dynactin motor occurs when RILP associates with the $p150^{(Glued)}$ subunit of the dynein motor effectively controlling the microtubule driven minus-end transport of late endosomes, lysosomes, and melanosomes [68, 69]. The *Salmonella typhimurium* vacuoles mature in a process similar to that of *Coxiella* in which it acquires Rab5 and Rab7 sequentially but do not fuse with lysosomes. Instead the vacuoles are relocated towards the cell periphery through a filamentous network due to recruitment of Rab7 by RILP [70]. Another intracellular pathogen *Brucella abortus* also resides in membrane-bound acidic compartment termed *Brucella*-containing vacuole (BCV). BCVs have been shown to acquire LAMP-1 and other late endocytic markers such as Rab7 and RILP indicating that the BCV development involves the recruitment of late endosome/lysosome components [71]. *Neisseria gonorrhoeae* reside in phagosomes inside host cells where maturation is arrested prior to acquiring Rab7 or RILP allowing the bacteria to thrive within the phagosome [72]. During *Coxiella* infection overexpression of RILP led to the formation of non-replicating CCVs suggesting that RILP maybe essential for CCV biogenesis [73]. Both RILP and Rab7 localize to the CCVs in ORP1L – depleted cells that resulted in smaller CCVs thus indicating that the RILP-Rab7 complex could be recruited independent of ORP1L and that the RILP-Rab7 complex cannot compensate for the role of ORP1L in CCV

size [45]. In order to better understand the role of RILP and the dynein-dynactin complex during WT and mutant *Coxiella* infection, a time course of the infection was conducted. RILP localization was visualized using immunofluorescent microscopy and scored by eye (Figure 10). Acquiring of the RILP across the 0 hpi to 72 hpi was delayed in the mutant CCVs relative to WT CCVs. Like the rapid recruitment of ORP1L following 12 hpi, RILP also displayed enhanced recruitment after 12 hpi in WT CCVs. Although mutant CCVs also displayed increased recruitment of RILP following 12 hpi, there was no increase in the number of RILP-positive CCVs between 24 hpi and 48 hpi. In contrast, >80% of WT CCVs were positive for RILP at 48 hpi and all WT CCVs were positive at 72 hpi. About half of the mutant CCVs do not acquire RILP by 72 hpi possibly indicating defective recruitment of RILP positive vesicles or aberrant interactions with the tripartite complex.

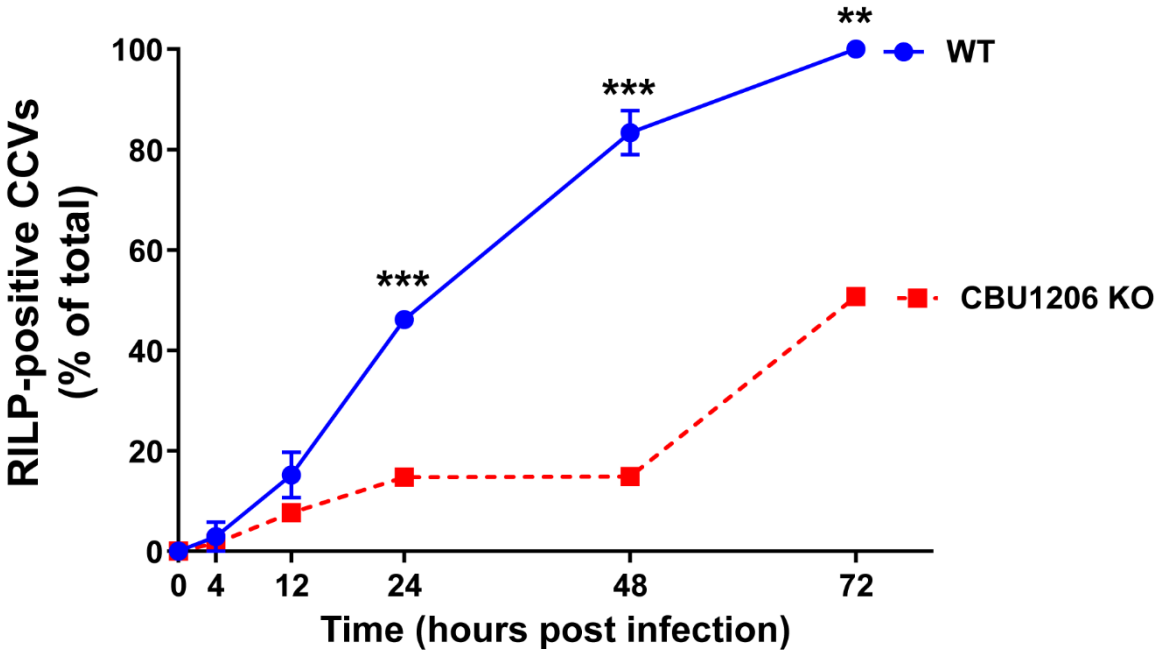

RILP-positive CCVs
(% of total)
100
80
60
40
20
0
0
4
12
24
48
72
Time (hours post infection)
**
***
***
WT
CBU1206 KO

Figure 10. RILP is Present on a Decreased Number of Mutant CCVs. RILP localizes to the CCV membrane of HeLa cells infected with (WT) mCherry *C. burneti* NMII Phase II. CCVs infected with mCherry *ΔCBU1206* displayed delayed recruitment of RILP. At 48 hpi, >80% of WT CCVs and <30% of mutant CCVs were positive for RILP. At 72 hpi, approximately 50% of mutant CCVs and 100% of the WT CCVs acquired RILP. At least 20 CCVs were scored as positive or negative for the presence of RILP. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

LC3 is Present on a Decreased Number of Mutant CCVs

Autophagosomes are suspected to have a pH ~ 6.0 and carry a broad array of cellular material to be targeted for autophagy. In this work, LC3 was used as the marker for CCV fusion with autophagosomes. Autophagy is a predominant response to stresses such as starvation and infection. Microtubule-associated proteins 1A/1B light chain 3B (LC3) functions in autophagy substrate selection and is a critical protein in the autophagy pathway. Hence, LC3 is a commonly used marker of autophagosomes. Intracellular homeostasis is closely tied to autophagy along with antigen presentation, infection, and cell death. LC3 containing autophagosomes are formed during the autophagy and fuse with lysosomes to form autolysosomes that degrade cellular components [74]. LC3 is also referred to as ATG8 and is one of the autophagy-related (atg) genes that regulate the autophagy pathway [75]. Several intracellular pathogens have been shown to subvert or manipulate the host autophagy pathway to acquire nutrients or xenophagy. *Listeria monocytogenes* once internalized survive inside macrophages in *Listeria*-containing phagosomes (SLAPs) that contain LC3 [76] possibly indicating that LC3 may be recruited for SLAP formation. In *Coxiella*, impairing host autophagy has resulted in a decrease in the overall number of CCVs and therefore, could be involved in the overall maintenance of the CCV [77]. LC3 has been shown to be acquired by the CCV as early as 5 min after infection and dependent on *C. burnetii* protein synthesis as no LC3 processing was visible in cell treated with chloramphenicol [78]. Furthermore, a screen of *C. burnetii* NMII transposon insertion mutants included those that had defects in effector proteins such as Cig57, CoxCC8, Cig2 and CBU1754. The authors reported a multi-vacuolar phenotype in mutants with transposon insertions in the Cig protein encoding gene. These vacuoles were also lacking LC3 while the WT CCVs displayed significant amounts of LC3 on vacuole [79]. Therefore, it is possible that the *C. burnetii* effector proteins secreted through the T4SS could be influencing the fusogenicity of *Coxiella* phagosomes and their ability to homotypically fuse with each other. In order to clarify the proper recruitment of LC3 to the mutant CCV and to understand the effects of autophagy following recruitment, LC3 localization on the WT

and mutant CCVs were visualized at 0, 4, 12, 24, 48, and 72 hpi. Cells were visualized using immunofluorescent microscopy and scored by eye (Figure 11). Interestingly, both WT and mutant CCVs display a pattern of localization similar to that of RILP localization. A dramatic increase in the localization of LC3 to WT CCVs was not observed at 4 hpi inconsistent with previous observations where LC3 localized 5 mins post infection [78]. This is most likely because the method of LC3 detection in these experiments is not sufficiently sensitive to detect the presence of small levels of accumulating LC3. By 48 hpi >50% of WT CCVs are positive for the presence of LC3 and 10% of the mutant CCVs were positive for LC3 recruitment. Mutant recruitment of LC3 seems to compensate somewhat at 72 hpi when the number of LC3-positive CCVs increase to approximately 40%. The 60% of mutant CCVs that are not positive for LC3 at 72 hpi might acquire the autophagy protein later in infection. The lack of it is indicative of defective autophagosome fusion or aberrant interactions of the mutant with the host autophagy pathway.

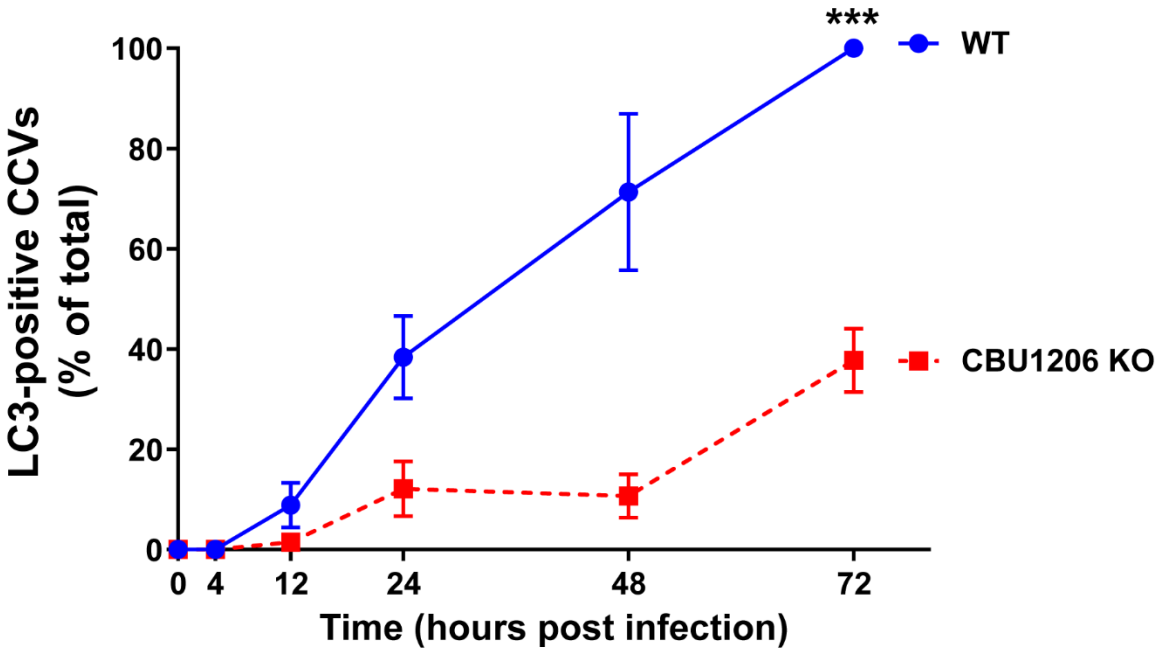

***
WT
CBU1206 KO
LC3-positive CCVs
(% of total)
100
80
60
40
20
0
0
4
12
24
48
72
Time (hours post infection)

Figure 11. LC3 is Present on a Decreased Number of Mutant CCVs. LC3 localizes to the CCV membrane of (WT) mCherry *C. burnetti* NMII Phase II infected HeLa cells. CCVs infected with mCherry *ΔCBU1206* displayed delayed and minimal recruitment of LC3. At 48 hpi, <30% of mutant CCVs and >60% of WT CCVs were positive for LC3. At 72 hpi, approximately 40% of mutant CCVs and 100% of WT CCVs were positive for LC3. At least 20 CCVs were scored as positive or negative for the presence of LC3. Results are shown for three individual experiments. Error bars represent the standard error of the mean. Statistical analyses were Multiple t tests and Holm-Sidak Method multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

ΔCBU1206 Possesses a Functional Type IV Secretion System

Often referred to as "adapted conjugation systems" Type IV Secretion Systems (T4SS) have homology to plasmid transfer systems. The Dot/Icm T4SS complex is possessed by intracellular pathogens such as *Legionella, Brucella, Bartonella, and Anaplasma* [80]. It is also possessed by *Legionella* related class member *Coxiella burnetii* [81]. The *C. burnetii* genome harbors 24 of the 26 *L. pneumophila* Dot/Icm proteins except for the DotV and IcmR proteins [82]. While the *L. pneumophila* T4SS has been shown to secrete 330 effectors that represents more 10% of the its whole genome, *Coxiella* secretes approximately 133 effectors that account for 6% of its genome [81]. These effectors have been demonstrated to influence a variety of CCV biogenesis events including expansion, nutrient acquisition, apoptosis inhibition, and manipulating host vesicle trafficking [83]. Effectors thought to influence host vesicle trafficking include CvpA that interacts with the clathrin-adaptor complex AP2 and is trafficked through the host endocytic recycling network possibly influencing host vesicle regulation [81, 84]. Effector proteins have also been shown to modulate the host apoptosis response to *Coxiella* infection. These include AnkG, CaeA, CaeB, and CBU1314 that block host mediated apoptosis through various mechanisms [81 {Weber, 2016 #188 {Weber, 2016 #188]. AnkG is a *C. burnetii* effector from the ankyrin repeat (Ank) family and it acts by binding to the host protein p32 to block pathogen-induced apoptosis [85]. CaeA seems to act on the apoptosis pathway by acting downstream of the cell death cascade and preventing the cleavage of caspase-7 that is responsible for execution of apoptosis. CaeB has been shown to target the mitochondrial outer membrane but the exact mechanism is unclear [86]. CBU1314 targets host driven apoptosis by associating with host chromatin to modulate the host transcription [87]. Further, mutants of the effectors CvpB, CvpC, CvpD, and CvpE were shown to have significant defects in intracellular growth and PV development with CvpB producing significantly smaller CCVs [88]. Unpublished data from the lab has also shown significantly smaller *ΔCBU1206* CCVs compared to WT. These observations and existing data on effector protein functions raised the possibility that the mutant phenotypes could be due to the lack of effector

proteins. Taking into consideration the substantial role effector proteins have during infection, it was critical to investigate mutant effector protein secretion capabilities. The translocation of effector proteins through the bacterial secretion systems has been monitored through Adenylate Cyclase Reporter Assays.

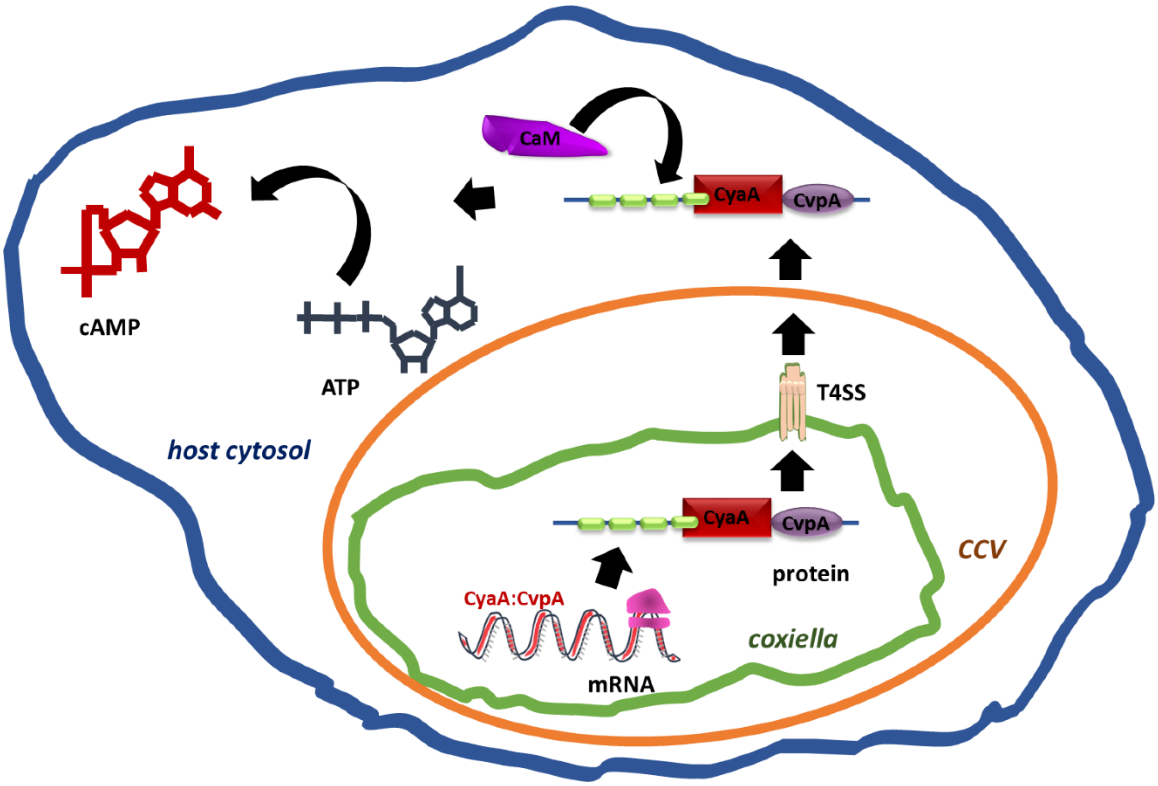

CaM
CyaA
CvpA
cAMP
ATP
T4SS
host cytosol
CyaA
CvpA
protein
CCV
CyaA:CvpA
coxiella
mRNA

Figure 12. The Adenylate Cyclase Reporter Converts ATP to cAMP. *CyaA:CvpA* strains produce mRNA that gets translated into protein inside the bacterium and gets translocated across the *Coxiella* Type 4 Secretion System. The calmodulin-dependent adenylate-cyclase domain of the adenylate cyclase (CyaA) reporter fused to CvpA encounters calmodulin (CaM) in the host cytosol. Upon binding of CaM with the ACD domain the adenylate cyclase converts cytosolic adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP).

For *C. burnetii* CyaA assays, transformants were constructed using pJB-CAT:BlaM and pJB-CAT:CyaA plasmid that were used to fuse BlaM and CyaA to the N terminus of C. *burnetii* effector genes [89]. We generated six different strains to approach mutant effector protein secretion as follows : *WT:CyaA, ΔCBU1206:CyaA, ΔdotA:CyaA, WT:CyaA:CvpA, ΔCBU1206:CyaA:CvpA,* and *ΔdotA:CyaA:CvpA*. The strains were fused with the adenylate cyclase or both the cyclase and *CvpA* that has been shown to delivered to the host-cell cytosol in a T4SS dependent manner [90]. The *CyaA*-only proteins should not be translocated to the cytoplasm as they are not fused to a known effector protein that is secreted. In contrast, the *CvpA:CyaA* proteins are translocated to the cytoplasm to produce cAMP, unless the T4SS is dysfunctional (Figure 12). The cAMP concentrations of the *CyaA* only strains were plotted over the *CyaA + CvpA* strains to generate fold changes in cAMP concentration for WT, *ΔCBU1206,* and *ΔdotA* (Figure 13). WT displayed the highest levels fold change of cAMP while *ΔdotA* displayed the lowest levels of fold change in cAMP concentration. The intermediate levels of cAMP fold change indicate that the *ΔCBU1206* does possess a functional Type 4 Secretion System even if it may not be as efficient as the WT T4SS.

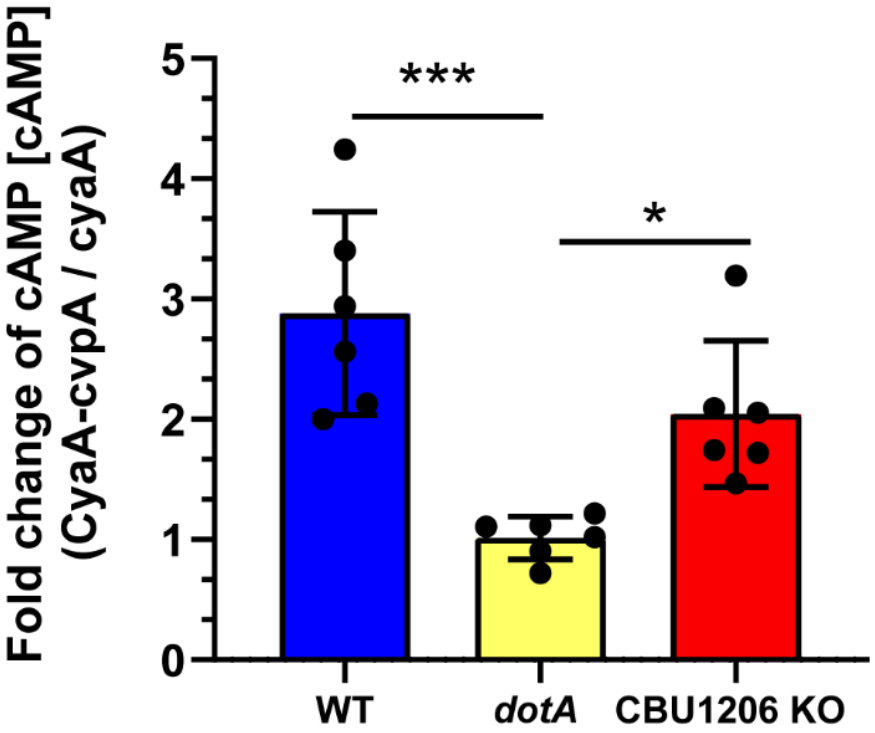

Fold change of cAMP [cAMP]
(CyaA-cvpA / cyaA)
5
4
3
2
1
0
***
*
WT
dotA
CBU1206 KO

Figure 13. *ΔCBU1206* Possesses a Functional Type IV Secretion System. *ΔCBU1206* has a functional Type 4 Secretion System. The fold change of intracellular cAMP levels was significant between *ΔCBU1206* and *ΔdotA* mutant. The fold change in WT cAMP levels was higher than both *ΔdotA* and *ΔCBU1206* KO. Results are shown for six individual experiments. Error bars represent the standard deviation from the mean. Statistical analyses were Ordinary one-way Anova and Tukey's multiple comparison test. * = $p < 0.5$; ** = $p < 0.05$; *** = $p < 0.005$; **** =$p < 0.0005$.

DISCUSSION

In this work, I examined the localization of markers for host endosomes and lysosomes to the *ΔCBU1206* CCV. This is an important aspect in the life of an intracellular pathogen because of the way these processes disrupt eukaryotic pathways. Since intracellular bacteria manipulate host cell vesicular transport pathways using diverse methods, it was important to investigate the localization of host proteins to gain a deeper understanding of *Coxiella*-host interactions.

Previous work from the lab demonstrated that *C. burnetii* inhibits endosomal maturation to regulate fusion with active lysosomes and maintain the ideal CCV pH (~ 5.2) [24]. This work also showed that increasing the number of proteolytically active lysosomes significantly stagger *Coxiella* growth and CCV development. However, even in the context of *C. burnetii* Δ*cvp* mutants, where the they lacked the *Coxiella* vacuolar proteins B, C, D and E individually, all mutants were positive for LAMP1 [88]. These mutants were shown to have defects in CCV development and intracellular replication similar to the unpublished findings related to *ΔCBU1206*. These findings are consistent with the data presented in this work where the mutant seems to preferentially fuse with lysosomes but is defective in CCV development. Observing the time course of LAMP1 localization to the mutant CCV, the pattern of localization is highly similar to WT where the marker is acquired rapidly and starting 4 hpi. Unlike the late endosomal markers LAMP1 was readily acquired by the mutant and might suggest that the *ΔCBU1206* CCV selectively fuses lysosomes and acidifies the vacuole. The selective fusion observed might lead to acidification of the vacuole and contribute to the growth defect observed (Figure 5B).

A recent study demonstrated that the CD63-positive intraluminal vesicles must be eliminated through lysosomal hydrolases for normal CCV membrane organization [88]. So, it can be predicted that CD63 is involved in a series of complex cellular events that may not be unilateral. According to our data, it appears that CD63 is recruited by the mutant beginning at 4 hpi and continues to be recruited at a steady pace until approximately 50% of the CCVs are positive for the marker at 72 hpi (Figure 6). In the

WT population CD63 localizes to 100% of the population past 12 hpi similar to the localization profile of the lysosomal marker LAMP1. It is unclear whether a percentage of the mutant population fails to recruit the CD63 positive vesicles altogether or fails to retain them. It may also be possible that WT fuses with a distinct vesicle population positive for both LAMP1 and CD63 but the mutant fails to acquire these distinct vesicles. Taking into account previous work from above, it seems that the *C. burnetii* carefully regulates the recruitment and elimination of CD63 vesicles through lysosomal hydrolases. So, it is also possible that the CD63 observed on the 100% of the WT CCVs at 12, 24, 48, and 72 hpi are CD63 vesicles that were retained while the rest were eliminated. This indicates a defect in the recruitment or elimination of CD63 vesicles by the mutant.

Cholesterol has been directly associated with the pathogenesis of numerous bacteria such as *Mycoplasma*, *Chlamydia*, *Anaplasma*, *Helicobacter* and *Borrelia* [91] [36]. They can manipulate the cholesterol trafficking pathways to access nutrients and membrane components that can ease vacuole formation. In *B. burgdorferi* this is thought to occur when there is direct contact between the bacteria and mammalian cells through outer membrane vesicles. The transfer of the outer membrane vesicles to host cells was assessed through the transfer of labeled cholesterol that have been predicted to form lipid rafts [91]. In contrast, cholesterol seems to play a negative role in vacuole formation of *Coxiella burnetii* where increasing CCV cholesterol acidifies the CCV leading to bacterial death [42]. Accumulation of cholesterol on the *ΔCBU1206* CCV membrane was shown (Figure 4A) and is possibly affecting the fusogenic properties of the vacuole. Furthermore, accumulation of cholesterol on late endosomes also results in the inhibition of intra-endosomal membrane dynamics altering protein and lipid trafficking [8]. I discovered cholesterol accumulation on the CCV and pursued this further by observing late endosome marker recruitment. Late endosomal markers Rab7, RILP, and ORP1L were scored on the CCV membrane.

The late endosome unique marker Rab7 was only present in 30% of the mutant CCVs at 72 hpi (Figure 8). This was surprising because Rab GTPases have been

established as a prominent method for intracellular pathogens to manipulate the phagosome to their advantage [88]. Although the WT slowly but steadily acquires the Rab7, the mutant lags and shows no increase in acquiring the marker starting 12 hpi. During phagosome formation Rab7 mediates fusion of phagosomes with late endosomes and recruits RILP in addition to the forming the tripartite complex (ORP1L-Rab7-RILP) [92]. The Rab7 effector RILP had a localization profile in WT CCVs similar to that of Rab7 where both markers localize to an increasing percentage of the CCV population from 12 hpi to 72 hpi (Figure 10). This might indicate defective phagosome maturation caused by defective recruitment of the dynein-dynactin motor complex since RILP has been shown to recruit this complex [68]. This directly impacts late endosome maturation, positioning, and other regulatory mechanisms that may be involved. The close association of Rab7, RILP and ORP1L might explain the similar localization patterns in WT CCVs. In the mutant CCVs Rab7 and RILP localization was dissimilar. Approximately 50% of the mutant population was positive for RILP while less than 40% of the population was positive for Rab7. Less than 50% of mutant CCVs acquiring Rab7 and RILP could be due to a critical flaw in the fusion of late endosomes by the mutant. If cholesterol is involved in CCV-host signaling this could indicate defective signaling related to the Rab GTPases as they are critical for endosomal maturation. Endolysosome vesicle maturation and differentiation could be defective in mutant infected cells.

The late endosomal marker ORP1L is present in less than 50% of the mutant and 100% of the WT CCVs at 72 hpi. ORP1L has been shown to bind the WT CCV membrane through N-terminal ankyrin repeats and simultaneously associate the CCV and ER [45]. Interestingly, the WT CCVs begin to exhibit ORP1L recruitment 12 hpi (Figure 9). It may be important to recruit ORP1L positive vesicles beginning at early times of infection considering its demonstrated importance in CCV-ER membrane contact sites and its role in endosomal maturation. This increase at 12 hpi was not observed in the mutant CCV population. Considering the effects of ORP1L depletion on CCV size, it is possible that the delay in acquiring late endosomal markers is due to lack of ORP1L at early stages of

mutant phagosome formation [45]. Further experimentation is needed to investigate Rab7-ORP1-RILP interactions and localization to the CCV. These observations suggest that approximately half of the mutant population is not acquiring endosomal pH and cargo possibly contributing to defective mutant CCV maturation.

Although, the role of LC3 has not been directly linked to CCV formation or *C. burnetii* replication, it is clear that autophagosomes fuse with the CCV. LC3 accumulates on the CCV as it expands and *Coxiella* replicates inside the CCV indicating fusion with autophagosomes [93]. I observed LC3 localization to the WT and mutant CCVs and observed a significant difference at 72 hpi only. In the WT CCVs, it appears that LC3 accumulates on the CCV as it expands based on the steady linear increase of the WT graph. This is in direct contrast with the mutant CCVs where <20% of the population was positive at 24 hpi and <40% was positive at 72 hpi. Our data suggests that the mutant is defectively fusing with autophagosomes and this may have larger implications in its ability to manipulate host cell autophagy.

A functional T4SS is essential for *Coxiella* to survive in a host cell past 2 dpi. This secretion system secretes effector proteins that modulate host cell processes and maintains the CCV. A persistent query at the heart of this study is whether the phenotypes observed with the CCV markers are due to a decreased number of effector proteins. I observed that the cAMP output of the *ΔCBU1206* was significantly higher than that of the *ΔdotA* mutant that is unable to secrete effectors (Figure 13). Yet, the amount of cAMP produced by the *ΔCBU1206* was approximately one fold less than WT indicating that although effector secretion occurs, it is not at endogenous levels. The limited effector secretion observed could contribute to the defective endosomal/lysosomal localization displayed by the mutant, but it is not the only causative mechanism. This reduction in the overall concentration of *Coxiella* effectors due to lack of CBU1206 might suggest that CBU1206 functions are essential for host cell manipulation and pathogenesis.

Fusion of the *Coxiella* containing vacuole with the host endocytic pathway is critical for the bacterium's survival and maintenance of the CCV as a replication-

permissive phagolysosome. This study examined the effects of host endosome/lysosome recruitment to the CCV in the absence of the putative sterol modifying enzyme CBU1206. The mutant defectively acquires the endolysosomal markers Rab7, ORP1L, RILP, and CD63. This effect was also observed with the autophagosomal marker LC3 indicating a defect in autophagosome fusion. In contrast, the mutant rapidly acquires the lysosomal marker LAMP1. In the absence of the CBU1206, there seems to be selective fusion with acidic vesicles such as lysosomes but minimal fusion with more alkaline vesicles such as late endosomes. Bacteriolytic effects have been observed during CCV cholesterol accumulation and CCV acidification [42]. Both sterol accumulation and CCV acidification might contribute to the defective CCV formation and fusion with host proteins.

A more comprehensive understanding of host protein localization to the developing CCV is needed. Experiments in transfected host cells visualized through live confocal microscopy could help discern whether host proteins accumulate or associate with the CCV membrane in a transient manner. Our observations of altered lipid content on the *ΔCBU1206* CCV membrane and this mutant being hypersensitive to host cholesterol (Figure 5) suggests this mutant can provide definitive conclusions in relation to the effects of sterol deregulation. Therefore, it maybe beneficial to observe the lumenal content of the mutant CCV for differences in vesicle fusion in response to increasing levels of cholesterol. Although *Coxiella* virulence is not directly attributed to CCV size or intracellular replication; altered lipid content in the CCV membrane might impact the CCV fusogenicity and viability in a host cell. Investigating WT and *ΔCBU1206* CCV formation and fusion events in a mouse model could provide insights into the impact of sterol deregulation on disease progression. Overall, these experiments would contribute to our knowledge on the topic of host and CCV cholesterol regulation by *Coxiella burnetii*. In this manner, the mechanisms of CCV biogenesis at the expense of host cell manipulation would be revealed.

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